Industrial scale composting process as a successful method for inactivation of potato cyst nematodes (Globodera spp. Behrens) and sugar beet cyst nematode (Heterodera schachtii Schmidt)

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Research Article

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Abstract

Cyst producing nematodes are persistent soil-born organisms causing severe damage to cultivated plants. Persistence of the economically relevant cyst nematode species Globodera pallida, G. rostochiensis and Heterodera schachtii was investigated at different stages during a large-scale industrial composting process to evaluate its efficiency to prevent spread of these nematodes into natural and agricultural habitats. Using reference cyst nematodes incorporated into organic waste from households and the processing industry the effect of anaerobic fermentation as well as aerobic composting processes were investigated. Treated cysts were analysed for viability and reproductive potential by performing hatching tests and bioassays on susceptible host plants. The investigated composting plant showed maximum temperatures between <40 and 72°C at aerobic composting conditions relative to the position of the pile the samples were incorporated. We found no viable juveniles or reproductive potential of Globodera spp. and less than five percent reproduction in H. schachtii. Additionally to temperature conditions, we presume that competition of the microbial community and their released bio-digestants also play a major role in successful treatment of these severe pest organisms.

Introduction

Besides rice (*Oryza sativa* L.), grain and maize (*Zea mays* L.) the potato (*Solanum tuberosum* L.) is one of the major crops worldwide (Marks, R. J., Brodie, B. B. 1998). Potato crops are used for direct consumption, supply of starch or animal feed. Sugar beet (*Beta vulgaris subsp. vulgaris convar. vulgaris var. altissima*) is of great importance in the food industry as one of the main sources of sucrose worldwide and could also be used as a high-quality animal food (Anonymus 2022).

Though a number of species of free-living nematodes and root knot nematodes cause damage to both crops, the potato cyst nematodes *Globodera pallida* and *G. rostochiensis*, as well as the beet cyst nematode *Heterodera schachtii*, globally cause major crop damage. Potato and sugar beet plants infested by cysts nematodes show primarily unspecific symptoms like stunted plants with yellowing or wilting leaves which finally die off if nematode pressure becomes severely high (Radtke et al. 2000). Infestation by cyst nematodes have been reported to cause yield damage as high as 60% in potato (Brodie and Mai 1989) and up to 25% in sugar beet industry (Daub 2021).

Potato cyst nematodes have their origin in Andean South America and show distribution rates of 14 and 22% of the annual monitoring area in Germany (0.5% of the potato growing production sites, data from annual reports to DG Sante; JKI). Both of the *Globodera* species are therefore under plant quarantine regulation in the European Union (European Commission 2022). Due to their endemic status Central Europe, beet cyst nematodes are not regulated as quarantine pests in Germany, but in some exporting countries such as South America, South Africa and Asia (Gamel et al. 2017). According to a definition by the Food and Agriculture Organization of the United Nations (FAO), the term “quarantine pest organism” describes pests or diseases of potential economic importance that do not yet occur or only to a limited extent in an area that is endangered by them and that are combated with official measures (FAO 2013).

The control of cyst nematodes is difficult due to the long persistence in the soil for up to 20 years (Grainger 1964, Been and Schomaker 1996, Turner 1996) and the existence of different pathotypes with different levels of virulence (Hockland et al. 2012, Niere et al. 2014). Commercial nematicides are in most cases no alternative to control cyst nematodes in field sites because of the uneconomic application with restricted efficiency lower than 30 percent (Greco et al. 2000) and the lack of registered compounds.

It has been shown that composting is suitable for killing harmful organisms (Bøen et al. 2006, Termorshuizen et al. 2005, Papajová et al. 2007). Composting is a process of biological decomposition of organic biodegradable material under controlled conditions that are predominantly aerobic. Microbial activity leads to the development of temperatures above 60°C so that the remaining solid particulate material becomes stable and sanitary free of most pathogenic organisms. The elevation of temperature is generated in the first mesophilic or maturation phase starting with temperatures around 20°C but not exceeding 40°C. In this phase of aerobic composting of crop residues and organic household waste the microbial activity is high (Zibilske 1998). Generally, the microbial activity is higher under mesophilic conditions at temperatures around 38–55°C than at temperatures of 60–70°C, where the most active groups of the microbiota necessary for the composting process are depleted from the complex microbial community. Above 70°C cyst nematodes cannot survive due to reaching their thermal death point (Womersley et al. 1998).

According to the legal standard requirements as stated in the German bio waste regulation (BioAbfV 2013) the treatment of biodegradable material should achieve uninterrupted thermophilic temperature conditions (55°C for 2 weeks, 60°C for 6 days or 65°C for three days) at a sufficient moisture (> 40%) and pH value (≈ 7) and sufficient nutrient contents for high biological activity (as defined in the BioAbfV 2013). Due to the humus cycle (re-use of composted household and industrial waste), the evaluation of the risk for spreading plant diseases by the use of compost that is processed in composting plants and then returned to fields is very important.

This study aims to determine whether the different processes of composting and fermentation could provide effective protection against the spread of viable stages of the cyst nematode species *G. pallida*, *G. rostochienis* and *H. schachtii*. Three independent analyses where implemented within regular runs of the composting plant Biokompostwerk (BKW) Bützberg close to Hamburg (Germany). Major experimental questions were (i) whether each of the processes (anaerobic fermentation and/or aerobic composting) could inactivate cyst nematodes, (ii) which temperatures were observed during the different process runs (iii) are there differences in process efficiency in order to achieve inactivation of the cyst nematodes between different seasons, and (iv) are there differences between samples rotated/not rotated in their position within the composting pile.

Cyst nematodes

All cyst nematodes undergo four juvenile stages until reaching their adult stage (Haque and Khan 2021). The life cycle of cyst nematodes starts when the second stage juvenile hatches from eggs. Hundreds of eggs are protected inside a single cyst, which is merely formed by the oxidised cuticle of the nematode female body. Second stage juveniles constitute the motile infective stage of cyst nematodes and the subsequent development takes place within the roots of host plants, where the movement of these juveniles is restricted to only a few inches or feet per year (Jenkins W.R., Taylor D. P. 1967). In the case of *H. schachtii* movement of second-stage juveniles from 30 cm up to 1 m almost in vertical direction within a few days has been observed (Westphal 2013). Hatching is induced by root exudates of the plant hosts in addition to adequate soil moisture, soil temperature above 5°C and oxygen availability (Marks, R. J., Brodie, B. B. 1998). While hatching of *G. pallida* and *G. rostochiensis* is predominantly dependent on the presence of potato root exudates (PRD) (> 70% of the population), a large proportion of hatching of *H. schachtii* can also occur in water while most of the population hatches in the presence of sugar beet root exudates (Jones et al. 1998). *H. schachtii* generally develops up to four generations per year depending on host availability (Perry and Wright 1998). It completes its life cycle within 31 days at 19 to 20°C (Jenkins W.R., Taylor D. P. 1967) while *Globodera* species need approximately 6 weeks for completing the life cycle unless development is ceased at temperatures higher than 25°C (Marks, R. J., Brodie, B. B. 1998). In most cases *Globodera* spp. shows only a single generation per year in the temperate soils of northern Europe (Marks, R. J., Brodie, B. B. 1998). Nevertheless, depending on host plant fitness, temperatures and day length up to 20% of the new eggs can hatch within the same year (Jones et al. 1998).

Materials and Methods

Fermentation and composting process

Experiments to test for the potential of aerobic composting processes as well as anaerobic fermentation in order to achieve inactivation of cyst nematodes were conducted at industrial scale in the composting plant Bützberg situated in the German federal state Schleswig Holstein. Operator of this plant is the municipal owned company Stadtreinigung Hamburg.

The fermentation and composting processes at the composting plant in Bützberg comprises several stages after delivery of organic waste (Fig. 1).

In most composting plants biodegradation of organic material comprises two main processes, anaerobic fermentation and aerobic composting. The fermentation and composting (depicted in Fig. 2) starts with the (i) preliminary separation of impurities (e.g. non-degradable contaminations such as plastic, metallic debris), shredding of the material and sieving through a mesh of 80 mm. Then the material is subjected to (ii) anaerobic fermentation and the remaining digestate will be entered into the (iii) aerobic post-treatment. The discontinuous dry fermentation process will separate the water (percolation) from the treated substrates in so called rotting tunnels to set up a compost pile (hybrid fermenter, Eggersmann Recycling Technology, Bad Oeynhausen, Germany). By changing the mode of operation and thus the process control, the hybrid fermenter can be used for both aerobic composting and anaerobic fermentation. Usual the material is composed of 80% organic waste after fermentation, 5% structure-forming green waste, 10% digestate for recycling and 10% processed sieve overflow. To enhance cost

efficiency of the composting plant biogas generated during fermentation (ii) is usually used for electric energy production.

After approximately one day in the fermenter the material reaches temperatures of about 40–42°C under anaerobic conditions for which the remaining air (N_2 and O_2) is expelled from the gas space (including the pore volume in the substrate pile) by CO_2 purging. The process takes about 15 days in total, then fresh air will be supply to start the aerobic phase for the subsequent composting process (iii), which will lead to further degradation of the material. The whole pile is converted into an empty hybrid fermenter in a weekly manner. Each time new material with coarse structure is used to aerate the material again. Temperature monitoring using temperature probes ensure that legal requirements are fulfilled (as stated in the German law BioAbfV 2007). The process normally takes three weeks with two repositionings, thereafter material is brought to the external warehouse. In the fine processing the compost is sieved through a 10 mm mesh, checked for quality and released for marketing. Coarser material is reused as structural material in the fermenter or used as fuel.

Multiplication of reference cyst material and sample preparation

Multiplication of reference nematode material (representative of populations mostly occurring in German field sites) and sample preparation was conducted at the JKI locations in Braunschweig and Elsdorf (Germany). The potato cyst nematode populations of *G. pallida* pathotype 2 “Kalle”, *G. rostochiensis* pathotype 5 “Harmerz”, and the beet cyst nematode *H. schachtii* pathotype Schach0 “MS” were used as references for testing the treatment’s efficiency in terms of nematode viability and reproductive potential over all phases of the bio-waste degradation process. Potato cyst nematodes were propagated on highly susceptible potato cultivar “Desiree” (Agropa GmbH, Brunnen, Germany). Tubers were pre-germinated at room temperature, planted in 9*9.5 cm plastic pots filled with loess with low nutrient content and free from plant parasitic nematodes. The loess was fertilised with 1.5 g of Osmocote long term fertiliser (Scotts Miracle Gro, Marysville; OH, USA) per kilogram loess. Nematodes were introduced into the pots using 1*1 cm nylon gauze bags with 100 µm mesh size, containing fifty nematode cysts, and placed close to the tuber. Cultivation was done in climate chambers with 16 hours light at a temperature of 21°C and 8 hours in dark with a temperature around 16°C. Water was added as required. Every pot was set into a separate coaster with 15 cm diameter to avoid nematode transfer after hatching. Finally, the plants were dried for five days and the sprouts were cut off. Newly formed cysts were separated from the substrate with a 200 µm sieve (Seinhorst 1964), dried and stored at 4°C for three months to simulate a natural diapause.

Beet cyst nematodes were propagated on highly susceptible winter oilseed rape cultivars in the greenhouse at JKI field station in Elsdorf. *Heterodera schachtii* J2 stages were acquired after submission of cysts for seven days in a 5 mmol $ZnCl_2$ solution to induce hatching. J2- stages were inoculated in pots with oilseed rape seedlings and cysts were harvested after development of two generations at

approximately 20°C. For experiments, 80 intact cysts of each cyst nematode species were sealed in gauze bags (100 µm mesh size), two individual bags for every treatment point.

Experimental design and introduction of cyst reference samples into anaerobic and aerobic fermenter systems

Three different experimental approaches were conducted to describe several scenarios providing a ground through situation of the processing of the plant in BKW Bützberg (Germany). As a standard procedure organic waste was first fed to fermenters (Fig. 2, ii) for biogas production and then transferred to the composting process (Fig. 2, iii) in large scale hybrid fermenters. *G. pallida*, *G. rostochiensis* and *H. schachtii* cyst samples passed through both processes successively. Since material may only be fed to either the biogas fermenter or the large-scale hybrid fermenter, samples were also placed separately in both fermenters in order to individually assess the efficiency to inactivate juveniles inside cysts. The reason for this separate investigation was that the material might just pass through composting solely in future as part of a short recycling process without preliminary fermentation. Therefore, the digestion of the material in the hybrid fermenter should also result in a product that is safe from a phytosanitary point of view.

The cyst samples were introduced by Planco-tec Analytic and Consulting (Neu-Eichenberg, Germany) into the pile of compost material using perforated stainless steel containers filled with the same compost material. Containers were incorporated into the pile at different positions (“bottom”, “middle”, “top”, Fig. 3) and attached to chains allowing easy repositioning in the compost pile. Temperature loggers (Gemini Typ TK-4014, Gemini Data Loggers Ltd., Chichester, West Sussex, United Kingdom)) within each of the steel containers recorded the process temperature.

In detail, three independent experiments were implemented with a duration of 3 to 4 weeks each. In a first setup in autumn 2020, only aerobic composting was analysed (8th to 29th October, Figs. 1 and 2) with placement of 12 cyst samples at three different positions of the compost pile (Fig. 3). This was done to elucidate the effect of sample position without turning the pile and elucidate the potential for blind spot development inside the hybrid fermenter. Blind spots with inefficient rotting conditions due to non-homogeneous rearrangement of the pile potentially appear at the edges where microbial mediated temperature increase does not reach the required minimum temperature to inactivate nematodes.

Under regular conditions, the samples were rotated weekly, together with the surrounding material, in an empty hybrid fermenter. Regular conditions were therefore investigated in a second approach. This approach was conducted during winter 2021 (18th February to 11th March with placement of the samples in a way that reproduces the turn mode together with its surrounding material, which is a standard procedure of industrial composting. Figure 3 shows the positions of the samples at the starting point of the composting run. Ideally, the samples and the surrounding substrate remain in the test positions (“top””, “middle”, “bottom”, see Fig. 3) that are present when introduced into the hybrid fermenter and rotate from front to back in the rotting tunnel when transferred.

In a third experiment, conducted in summer 2021 (8th July to 3rd August), one series of reference samples comprising 4 replicates for each vertical position was incorporated into the whole process of anaerobic fermentation and aerobic composting and a second set of 12 replicates was separately subjected to fermentation or composting. Fermentation was conducted for 15 days at the end of June 2021 without further processing of composting (Fig. 2) and the second subset of samples passed through the whole process (fermentation + composting) and was therefore additionally treated for another 27 days in the subsequent composting run of the organic material.

Hatching Test and Bioassays

To measure the viability of the treated cysts a hatching test according to EPPO Standard PM 7/40(4) (EPPO 2017) was used for potato cyst nematodes. Twenty cysts were randomly chosen out of each gauze bag and 1 mL hatching medium (potato root diffusate, PRD; obtained from the susceptible potato cultivar “Desiree”, stored at 4°C) was added. The samples were incubated at room temperature in 10 mL glass vials with a snap-lid. The number of hatched juveniles were counted in weekly intervals using a stereo zoom microscope (Wild Heerbrugg, Heerbrug, Switzerland). The hatching test was performed over seven weeks and PRD was changed each week.

For beet cyst nematodes, 20 cysts were randomly isolated form gauze bags and incubated on petri dishes filled with 5mmol $ZnCl_2$ solution for 14 days at room temperature. Juveniles were separated using a 20µm sieve and counted under a stereo microscope (Leica MZ APO, Wetzlar, Germany) at 50x magnification. Juveniles that did not hatch and inactive eggs were separated from remaining cysts using a cyst homogenizer and released juveniles and eggs were counted.

To determine whether there were differences for the number of hatched juveniles and the production of female adult nematodes in the population, bioassays were conducted from end of March to the beginning of July 2021 and from the mid of February to the end of May 2022. For bioassays of potato cyst nematodes the procedure described by Kort et al. (1977) using nematode cysts instead of hatched juveniles was applied. Therefore the ratio of new cysts produced (P_f) to the number of cysts added initially (P_i) was calculated. Whole cysts were used from the replicates of each position in the three composting runs and compared to untreated controls from the same series of reference cyst multiplication (2018/2019). In the case of *Globodera* spp. the bioassays were conducted under the same parameters as described for the production of reference nematode cysts stated above. Newly developed cysts were counted with a stereo zoom microscope (M8, Wild Heerbrugg, Germany) at ten times magnification.

In case of *Heterodera* gauze bags (containing either fifty treated or untreated (control cysts) were placed in a 400 mL pot and filled with 350 g of loess enriched with fertiliser (3g Kg^{-1} Osmocote Exact 3–4 M). Four seeds of a susceptible oilseed radish (*cv*. Siletina) were seeded per pot and thinned to two remaining plants after seedling emergence. All pots were drenched with 30mL of the fungicide Previcur (1.5 mL L^{-1}, 530. g L^{-1} propamocarb, 310 g L^{-1} fosetyl) per pot to prevent seed born damping off disease. All variation

of combined vertical and horizontal positions plus untreated controls were set up as complete random blocks. Greenhouse temperature was set to 20°C/16°C (day/ night temperature) for 12 hrs each in additional light (2700K). Data logger continuously recorded soil temperature and test was terminated after 390 degree days (days with temperature higher than 10°C; the sum was calculated by adding the daily temperature minus 10 degrees per day) allowing reproduction of one *H. schachtii* generation. This was monitored by specific controls with untreated cysts that achieved reproduction rates using the ratio of new cysts produced to the number of cysts added initially $P_f/P_i > 2$. After removing original gauze bags used for inoculation, newly developed cysts were extracted from loess in individual pots using a sieve combination (1000/100 µm) and a subsequent centrifugal flotation method (Hallmann et al. 2021). Cysts were counted and contents (juveniles and eggs) were released using a cyst homogenizer. Juveniles and eggs were quantified per 100g of soil for each sample using a stereo microscope at 50x magnification. Reproduction rate was calculated from P_f to P_i relation, where P_i was the total number of nematodes in 50 cysts for inoculation.

Statistics

Data of the hatching tests and bioassays were analysed using non-parametric Kruskal-Wallis tests with pairwise Dunnett's post-hoc test and subsequent type I error Bonferroni correction of post-hoc test results (Sokal and Rohlf 2012) as implemented in R-package MultBiplotR (Vicente-Villardon 2021) running under R Version R-4.03 (R Core Team 2021). Normal distribution of daily temperatures were confirmed by using R-function "hist" and Shapiro-Wilks test and therefore analysed by ANOVA (analysis of variance) with Tukey's post-hoc test for pairwise comparisons of composting run season and treatment position in the pile using R. Student's t-tests, as implemented under R-basic package stats, were used to explore the differences in hatching test controls between the composting runs conducted in autumn 2020 and summer 2021.

Results

The processes of anaerobic fermentation, aerobic degradation of the organic material by composting and the complete process of fermentation and composting as routinely conducted at the composting plant Bützberg were analysed separately for their efficacy regarding inactivation of cyst nematodes. In one out of three composting runs conducted in summer 2021, anaerobic fermentation with a reduced residence time of the material of 14 to 16 days in comparison 25 days in "conventional" biogas plants as well as additional fresh air exchange by means of CO_2 flushing was included in our analysis. Anaerobic fermentation was the second step of the whole recycling process after sorting. A subset of twelve samples passed solely through fermentation while the remaining twelve samples passed through the whole process of fermentation and subsequent composting. The temperature in the fermenter was observed to be constantly between 40–42°C during the whole treatment. The results of the hatching tests of juveniles showed that using only fermentation is sufficient for successful inactivation of cyst nematodes (Fig. 4A). The same result was observed for these samples passing through both of the processes (Fig. 4B).

We observed significant differences in temperatures reached in aerobic composting conducted in winter 2021 compared to the both runs in autumn 2020 and summer 2021 (Fig. 5). This was due to the fact that the oxygen rich air was additionally temperated with exhausted air from a warehouse, whereby without additional heating of the air, seasonal fluctuations ultimately also occur. Temperatures at the three different positions varied significantly, generally being highest at the top and lowest at the bottom (Fig. 5).

In autumn, the temperature profile was clearly distinctive between the vertical levels with maximum temperature at Top position of higher 65°C for 6 days and maximum temperature at basis level 40–42°C for 6 days. The daily temperature amplitude between lowest temperature measured at the Bottom position and highest at the Top position varied between 19.3°C and 68.1°C. The clear temperature stratification between the layers was not given in the other composting runs. In winter, the highest temperature was measured at the Bottom position with 72.4°C, followed by the Top position with 69°C and 64.1°C at the Middle position. The daily temperature amplitude varied between 32.2°C measured at the Top layer and 72.4°C. The lowest maximum temperatures of the examined composting runs were reached in the summer of 2021. The maximum temperature measured in the top layer was 62°C, followed by the Middle layer at 57.2°C and the bottom layer at just 43.8°C. The minimum temperature was measured at 22.9°C in both the bottom and middle layers. Over all composting runs, the discrepancy for daily temperatures at the horizontal level in the rotting tunnel belonged very low (Fig. 6).

Hatching tests for three independent runs of composting showed that no hatching of juveniles was found for both *Globodera* species as well as for the beet cyst nematode *H. schachtii*. This was observed for composting runs conducted without proceeding anaerobic fermentation.

Since there could be a potential risk that blind spots of untreated material can occur within the compost pile, resulting from non-homogeneous turning, it was examined in one experiment in October 2021, if turning the material enhances the efficiency of the composting process. Material was rotated according to the routine procedure, but samples were placed by hand at the same position where they were placed in the beginning. No influence of turning the material was detected and efficiency of cyst inactivation was 100% (Fig. 7C).

In order to better understand morphological changes in cysts, eggs and juveniles affected by the treatment in relation to the non-treated control we used dissection (up to 100x magnification) and light microscopy (at 400x magnification, see Fig. 8). It was observed that the colour of cuticula in the treated cysts had changed from light brown to extremely dark brown to black compared to the control cysts. The content of the cysts was mainly destroyed and most of the unhatched eggs were found to contain a brownish mass (Fig. 8D, middle). After cutting of the cyst and removal of the eggshell by slight pressure, juveniles showed characteristics of mortality, like vacuole formation in their abdominal intestine and body deformations. Over time, the content of eggs darkened and the collapse of second stage juveniles could be observed.

In concordance to hatching tests, the bioassays showed no reproductive activity for the potato cyst nematode species at all positions in the composting pile and in all three composting runs (Fig. 7). Nevertheless, some reproduction (4%) for the treated cysts of *H. schachtii* could be observed, with resulting P_f/P_i between 1.00 and 1.04 for samples from the middle position during the winter run. Compared to the control samples of *H. schachtii*, which showed multiplication rates of 17 and higher, the multiplication observed in treated samples was minimal and acceptable under phytosanitary aspects.

Hatching of juveniles per cyst in controls of *H. schachtii* remained relatively constant during all seasons ($t = 0.088518$, $df = 4.1572$, $P = 0.9336$). Whereas hatching of juveniles of *G. pallida* decreased from 224 juveniles per cyst in the autumn composting run to less than 17 hatched J2 in summer 2021 ($t = 18.9$, $df = 2.5081$, $P < 0.001$) in relation to approximately 500 eggs present per cyst (Fig. 7). This effect could also be observed for *G. rostochiensis* with a lower rate of decrease ranging from 170 in autumn 2020 to 57 in summer 2021 ($t = 12.692$, $df = 2.3528$, $P = 0.0032$).

Discussion

A recent risk based assessment presented by the Norwegian Scientific Committee for Food and Environment (Alsanius et al. 2021) globally analysed the reliability of pathogen treatments in biogas and composting plants in order to avoid spread of pathogenic organisms into natural and agricultural habitats. This study completely depended on literature data and concluded that potato cyst nematodes are expected to completely withstand both, aerobic mesophilic fermentation and anaerobic mesophilic digestion as well as vermicompost processes and basket composting. In conclusion, infectivity and reproduction remain to be investigated especially with regard to composting in actively and passively aerated piles. Therefore, survival rates will depend mainly on the temperature reached and duration of high temperature conditions. The risk analysis estimated a treatment with temperature within the pile of 55°C for four weeks not to be sufficient for eradication of nematodes from genera *Globodera* sp. and *Meloidogyne* sp..

In contrast, the results in our study showed that both, the composting process in the hybrid fermenter and the anaerobic fermentation process are highly efficient methods to inactivate cyst nematodes and thus avoid spread of these pests. We proved viability and infectiveness for reference samples of the cyst nematodes *G. pallida*, *G. rostochiensis* and *H. schachtii* introduced into mesophilic to thermophilic aerobic composting and mesophilic fermentation processes at the composting plant Bützberg. Mesophilic fermentation with temperatures below 50°C was analysed independently from aerobic composting in summer 2021. Temperatures around 40–42°C were achieved during this process and were found to be sufficient to successfully inactivate eggs inside the cysts by 100%. This confirms the results of Spaull (1989) who observed a complete loss of viability of the eggs from *G. rostochiensis* and *G. pallida* in laboratory experiments during anaerobic fermentation within 30 min at 35°C using sewage sludge or 9 weeks without temperature elevation (cold anaerobic digestion). In numerous other recent studies, this effect could be attributed to the production of nematode suppressive by-products during microbial fermentation processes (Eberlein et al. 2019, Jothi et al. 2003) rather than an elevation of the

temperature. Nevertheless, the identification of secondary metabolites with nematicide effects and their practical or commercial use remains difficult due to the high variability of the organic source material and the variation of operating parameters during fermentation (Oldani et al. 2022, oral presentation). Besides O_2-depletion a number of volatiles e.g. volatile fatty acids (Davis et al. 1997), other organic acids and to some extent elevation of CO_2 and NH_3 seem to have a relevant impact on nematode inactivation while others (e.g. CH_4 and H_2S) could not be correlated with such effects (Runia et al. 2014). In sum, the anaerobic conditions, CO_2 elevation and microbial secondary metabolites (digestates) with potential nematicidal effects provided a proper control of cyst nematodes within a rather short period of 10 to 15 days in our analysed fermentation run.

The temperatures in the composting process during our study reached between 40–70°C and therefore minimum temperatures for effective treatment were lower in some areas of the composting pile than previously recommended. The test design used in our analyses allowed for a differentiation of the temperature effects in the different zones of the pile (top, middle, bottom), with the lowest mean temperature being observed in the bottom area. The highest temperature was found in the top zone and not as expected in the core. These findings differed between seasons, during autumn and summer the differences between the rotting zones were significant while the mean process temperature was most constant during the run conducted in the winter 2021 with no significant differences between the rotting zones (Fig. 5). Daily measured temperatures in the autumn composting run showed the highest differences in their amplitude within each of the vertical levels accompanied by a clear vertical stratification of the layers in the composting pile (Fig. 6). This shows the huge variability in process regulation and the importance of repeated analyses for phytosanitary control of the processes in such plants.

In the bottom rotting zone, temperatures did not exceed 42°C in the composting runs conducted in autumn 2020 and summer 2021 which should, according to previous literature (Wallace 1963, van Loenen et al. 2003, Alsanius et al. 2021), not be sufficient to inactivate cyst nematodes without turning of the material. In the literature, a number of partially conflicting studies can be found which were mostly conducted on cysts of *G. rostochiensis*. Wallace observed that cysts of *G. rostochiensis* survive less than one hour when submerged in water warmer than 47°C and van Loenen showed that less than 3 minutes in 60°C aerated steam are not sufficient to inactivate cysts of this species safely (Wallace 1963, van Loenen et al. 2003). Furthermore, there is a difference between soaked cysts and dry cysts. While pre-soaked cysts contained no viable eggs and juveniles after 30 minutes at 58–59°C (Evans 1991) dried cysts required temperatures of 90°C for more than 30 minutes or 65–70°C for up to 24 h for inactivation (Lindhardt 1959, Stone and Wesley 1975). Reports on the effect of temperature on cyst nematodes within the composting process are scarce and inconsistent. The Soil Association Standard for Organic Farming and Production (Soil Association, 2002)(Soil Association 2002)emphasises the beneficial effects of composting to reduce pathogen loads and to produce a product that is stable in pH-value and not further degradable or showing any phytotoxic effects. The authors of this standard recommend a temperature higher than 55°C for at least three days, achieved by turning the material and supplying forced oxygen

enriched air to destroy most weed seeds, pathogens, chemical residues and antibiotics. Another study (Gale 2002) suggests a minimum of 60°C for two days where composting piles should be turned at least three times within 14 days during the composting process. Christensen et al. (2002) reported that composting requires 70°C for two days or 65°C for four days with at least five turns of the pile, and Nishinome and colleagues (1997) showed composting at 40°C for ten days or at 50°C for five days is sufficient for the inactivation of cyst nematodes.

If ventilation of oxygen rich air is not optimal, unfavourable fermentation residue conditions or too little structure, anaerobic stains can occur in the compost heap. However, this would be noticeable through low temperatures. Therefore, these initial factors play an important role in the process but it is largely ruled out by the weekly moving and ventilation. Turning the pile enables oxygen flow to avoid anaerobic gaps accompanied by temperatures lower than 45°C of up to 20% of the composting mass (Noble and Roberts 2004). We analysed the effect of turning versus not turning of the pile in autumn 2020 and did not see any differences in terms of cyst nematode inactivation between the runs.

In case of the composting process, some important process parameters recommended in the literature regarding temperature and retention time of the organic material in the hybrid digester were not fully achieved, but the treatment of the cyst nematodes was nevertheless successful. Fermentation and composting in an aerobic hybrid fermenter are parts of the same process chain as conducted in the composting plant Bützberg. This means that the resulting material from anaerobic fermentation will contain numerous nematicidal organic compounds as described above and will subsequently be used for aerobic composting to produce a fine-grained finished compost at the end. The hypothetical presence of soluble nematicidal compounds in the starting material for composting could explain the successful inactivation of cyst nematodes by daily temperatures as low as 40°C. Therefore, effects of anaerobic and aerobic processes cannot be disentangled.

The different nematode species showed slight differences in their ability to endure temperature. It is known from the literature that *G. rostochiensis* is more heat-resistant than *G. pallida* (Stone and Wesley 1975). *H. schachtii* has been reported to be able to persist in the first maturation phase for up to six weeks at temperatures lower than 55°C. Only in the thermophilic sanitisation phase at higher temperatures they die (Noble and Roberts 2004). Although there remained a few hatched juveniles from *H. schachtii* not able to start population growth in absence of the sugar beet host, the composting processes tested here were safe in relation to the deletion of the tested reference cyst nematode species.

Hatching tests and bioassays are routine testing procedures to investigate viability and infectiveness of nematodes (for *Globodera* sp. concluded in the standard PM 7/40(4) EPPO 2017 and for *H. schachtii* see Whitney 1970). The number of hatched juveniles and reproduction in bioassays of non-treated controls decreased for potato cyst nematode species but not for *H. schachtii* ongoing in time when the experimental runs were conducted (Fig. 7). For bioassays the ratio of new cysts produced (P_f) to the number of cysts added initially (P_i) was calculated. The remaining factor of population growth in bioassays in the presence of a susceptible potato host and natural light remained constant but could not

be directly compared with the results of hatching tests. In most cases, hatching tests were conducted off-season, which means not under natural hatching conditions in autumn and winter. Therefore, a number of endogenous factors could affect hatching success. With increasing time of storage of the controls at 8°C the probability that the cysts undergo dormancy increases, which results in a long term downregulation of the cyst metabolism not easily reversible (Jones et al. 1998). However, the differences between control and treated cysts are statistically significant in all cases and provided proof of successful treatment of compost during composting and anaerobic fermentation in the composting plant Bützberg.

Based on the results generated in the present study, we can assume that maximum inhibitory effects can be achieved at low temperatures of < 50°C and that other factors, not analysed in our study as microbial activity could play a key role. Based on the literature, this would also be true for seeds of weeds and harmful organisms. We conclude that at this temperature level the risk of compost matter for the spread of PCN and BCN into environment is negligible from a phytosanitary point of view. In consequence, numerous factors as organic material composition in interaction with process control both responsible for microbial community and subsequently bio digestants composition have to be considered for a safe composting process. Our study showed that a safe product in regards to *G. pallida, G. rostochiensis* and *H. schachtii* could be produced with our test conditions in the composting plant in Bützberg. Further investigations are needed to understand which specific conditions should be used for sterilising processes during composting of biodegradable material.

Declarations

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Conflicts of Interest*:* The authors declare to have no conflict of interest.

Clarification of authorship: All authors agree with the content of this publication and consent to submit this article to the journal. The authors declare that they obtained content from the responsible authorities of the Julius Kuehn Institute for the article. The authors declare that they have no conflict of interest with the data published here. All authors whose names appear on this submission made substantial contributions to the conception or design of the work or interpretation of data.

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Figures

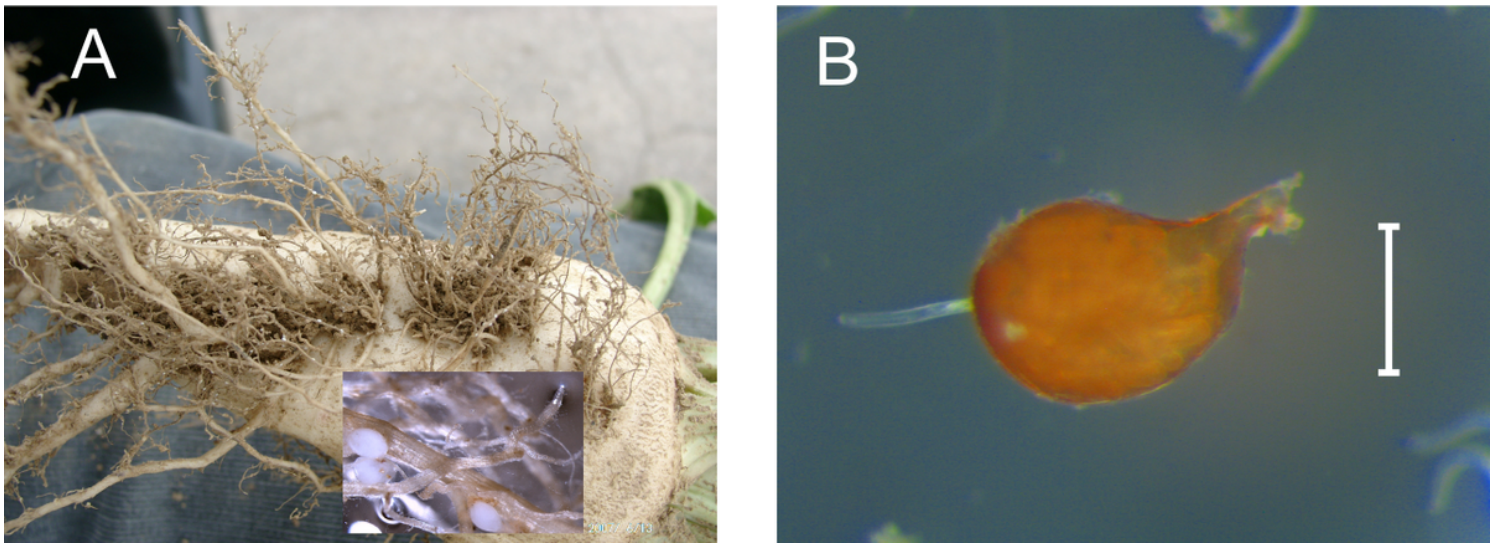

Figure 1

Figure 1a: (A) nematode cysts on fine roots of sugar beet and young developing females in detail; (B) second-stage juvenile of Globodera rostochiensis hatching from cyst measuring 0.3 mm under 100x magnification.

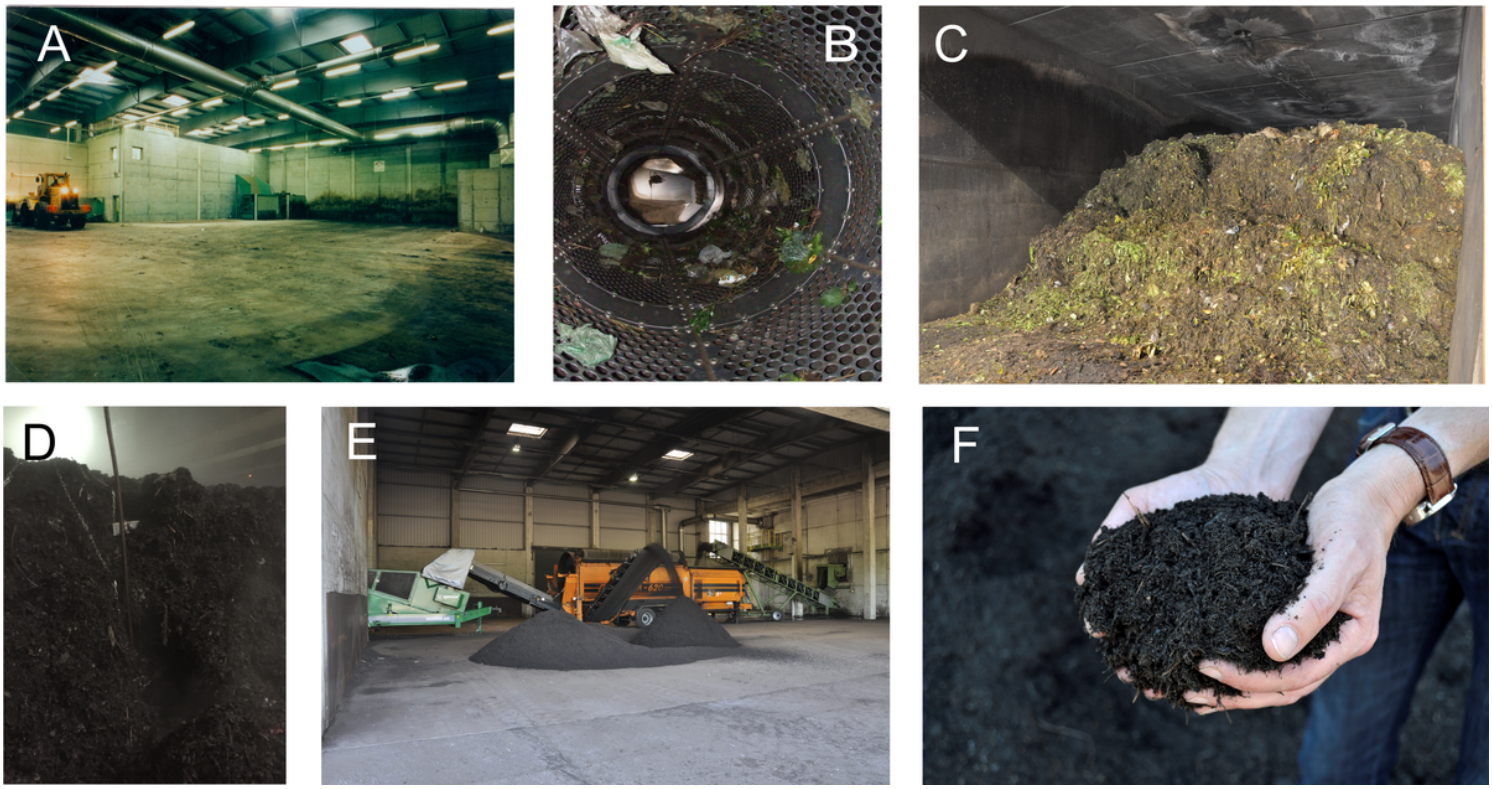

Figure 2

Figure 1: Pre-treatment and fermentation (A-C), and composting (D-F) processes in composting plant Bützberg: A) charging hall, B) sieving and separation of foreign matter, C) fermentation, D) composting process in the hybrid fermenter, E) sieving, F) finished compost product

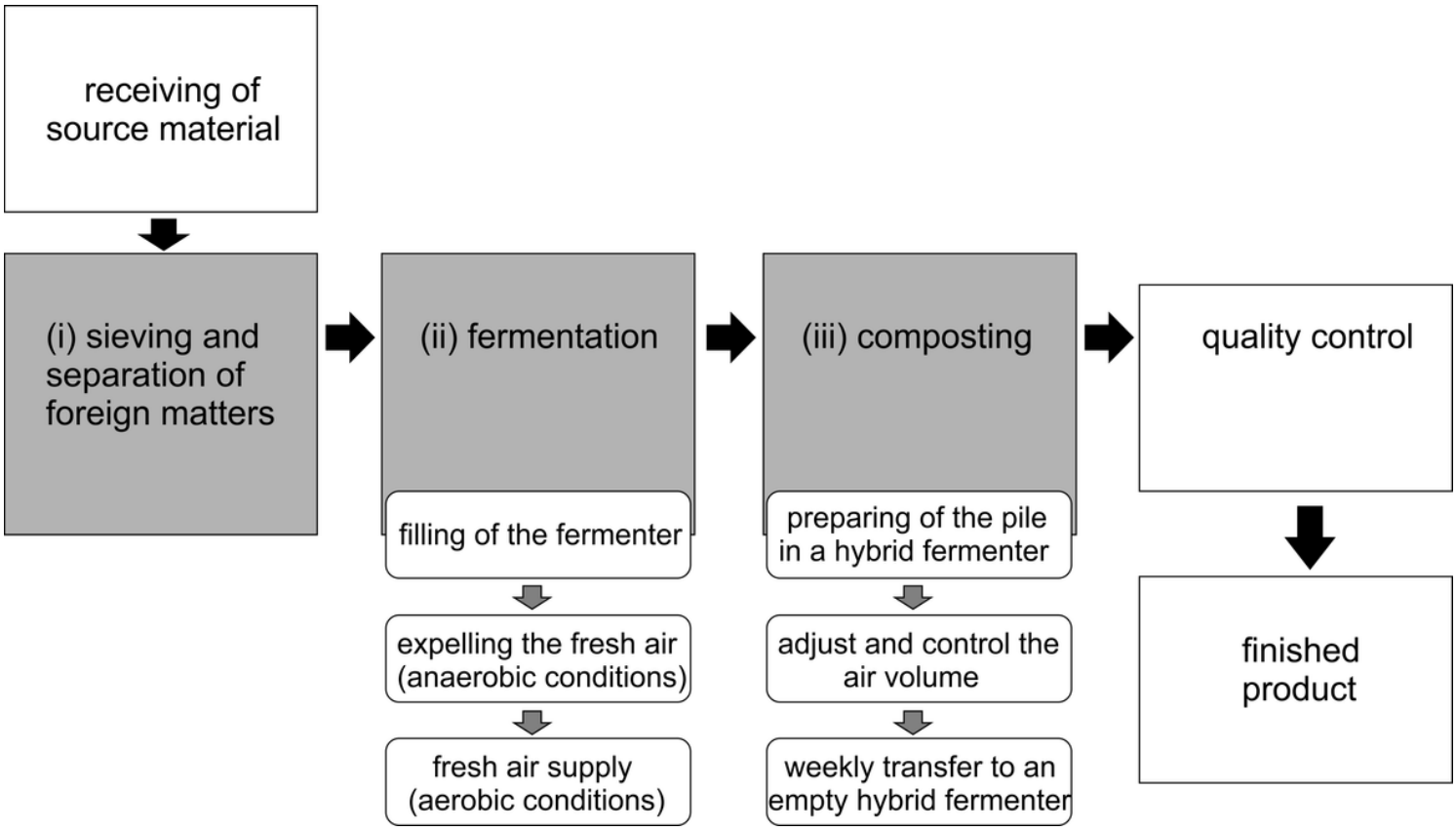

Figure 3

Figure 2: Workflow of the processes in the composting plant Bützberg

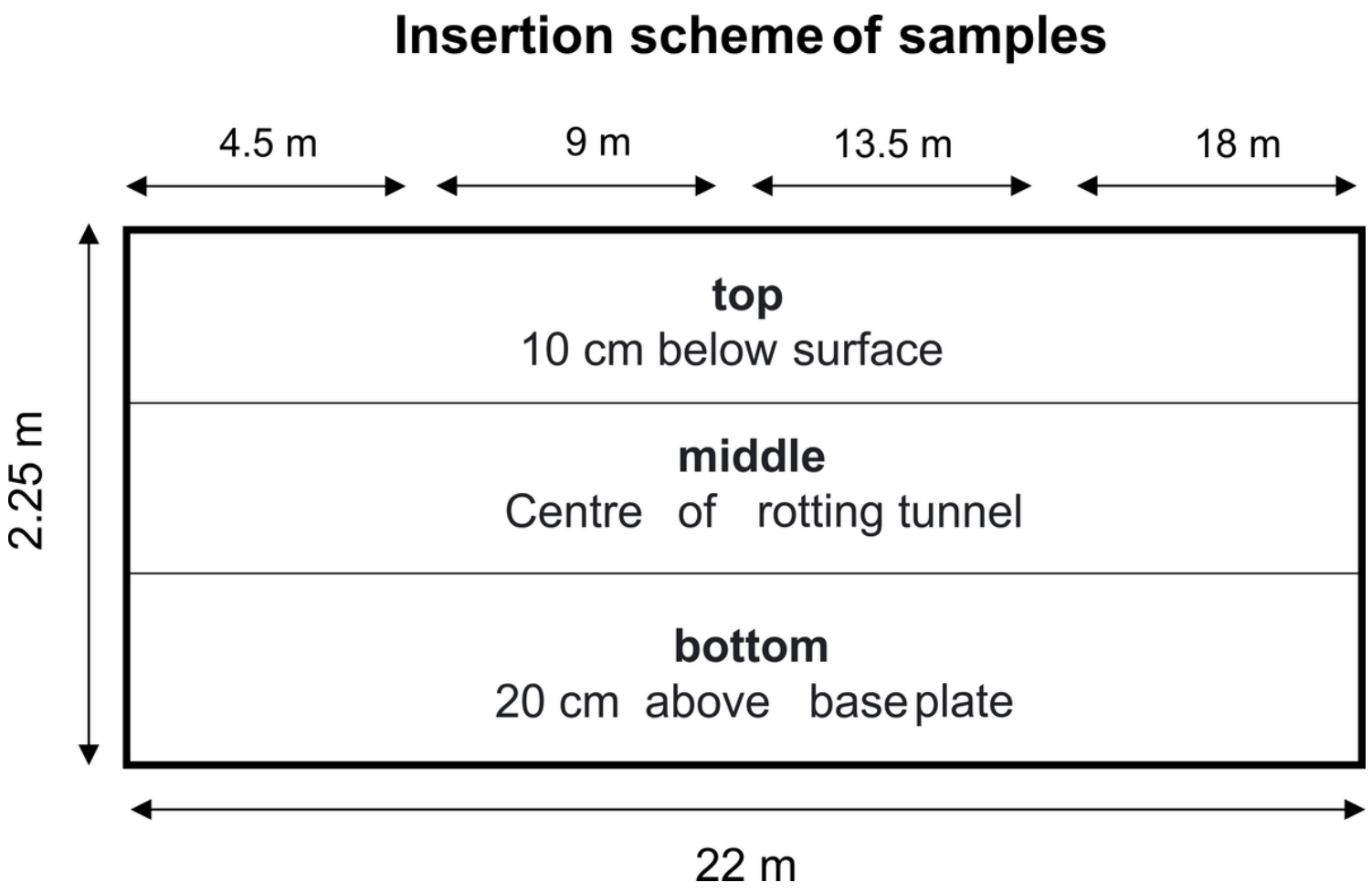

Figure 4

Figure 3: Schematic drawing of the hybrid fermenter used for composting of organic waste. A total 12 samples were placed on four horizontal positions (arrows above) in each of the three vertical rotting zones top, middle and bottom with two gauze bags in each combined position

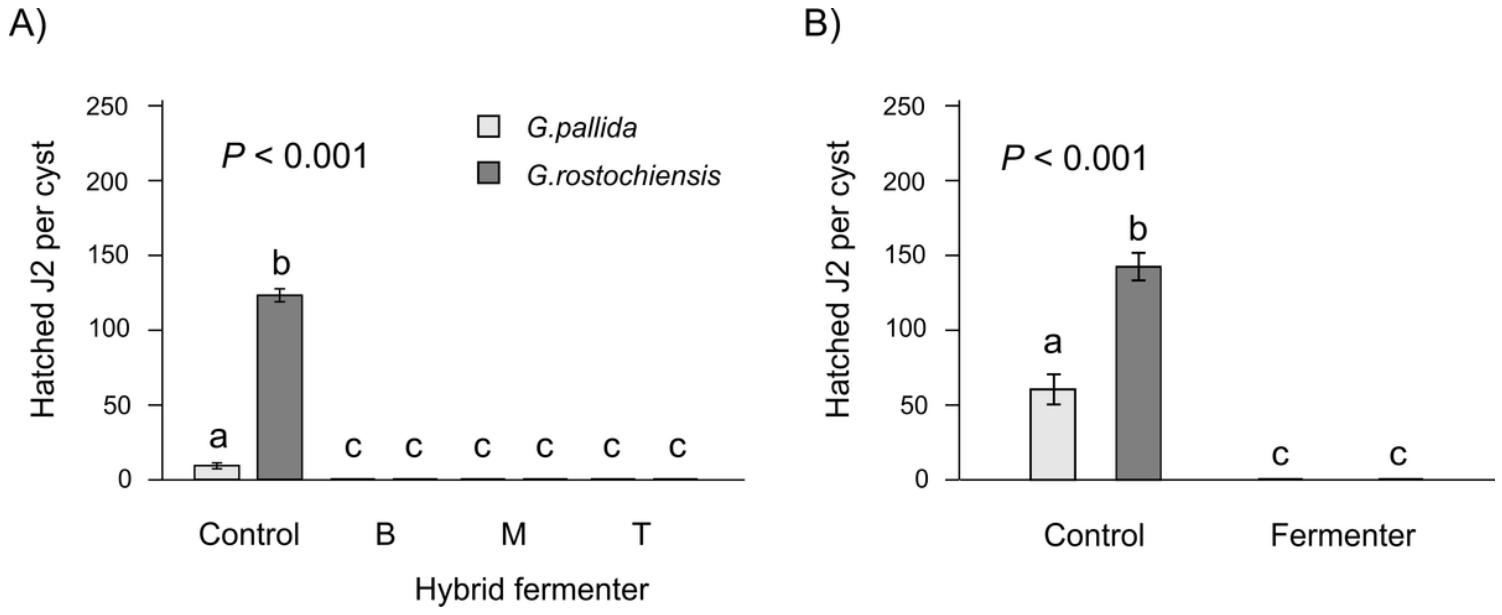

Figure 5

Figure 4: (A) Hatching of juveniles of *Globodera pallida* and *G. rostochiensis* from cysts passing through the complete process of fermentation and composting (n = 4 replicates per position), and (B) from cysts exposed only to the Fermenter (n=12 independent replicates) for 15 days. Twenty cysts (<500 µm) per recovered gauze bag and position in the Hybrid fermenter (B = bottom, M = middle, T =top) were tested for hatching. Technical replicates of the control (20 intact cysts, <500 µm) were also set up (n = 3) and standard error is shown as whisker.

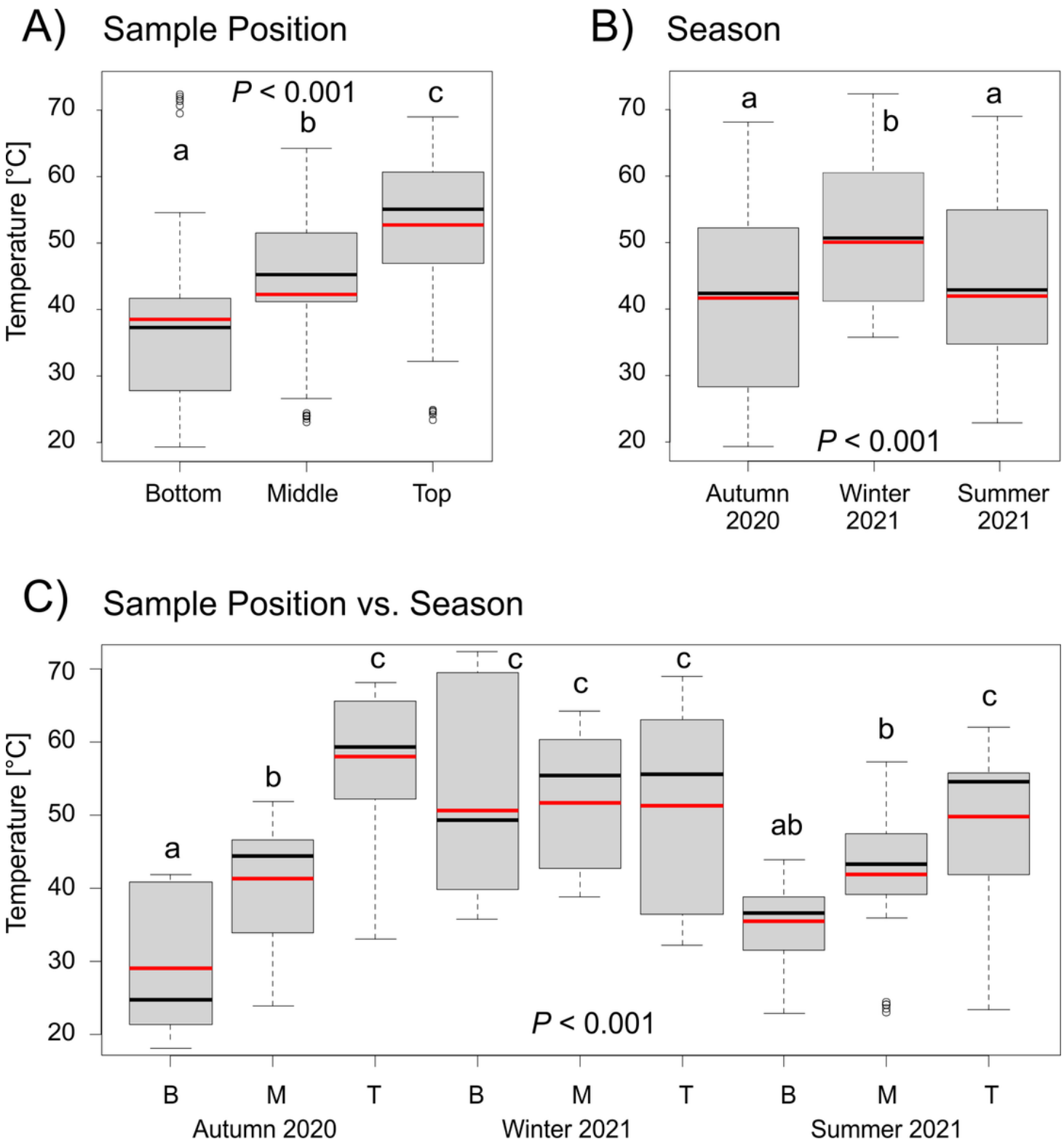

Figure 6

Figure 5: Box and whisker plots of daily temperatures measured in four replicates for each sample position, bottom, middle, top. A) Temperature in all three composting runs for each sample position; B) temperature at all positions for the three different seasons, and C) combination of position and season. Box and whisker plots show the median of temperatures (black bold horizontal line), mean (red bold horizontal line), and 25th and 75th percentiles (bottom and top of the box), while whiskers (vertical dashed

lines) represent approximately the twofold standard deviation. Circles indicate potential outliers. Results of analyses of variance (ANOVA) are shown for each of the analysed comparisons and differences between the sub-groups calculated by Tukey's post-hoc tests are given in different lowercase letters above each box and whisker plot

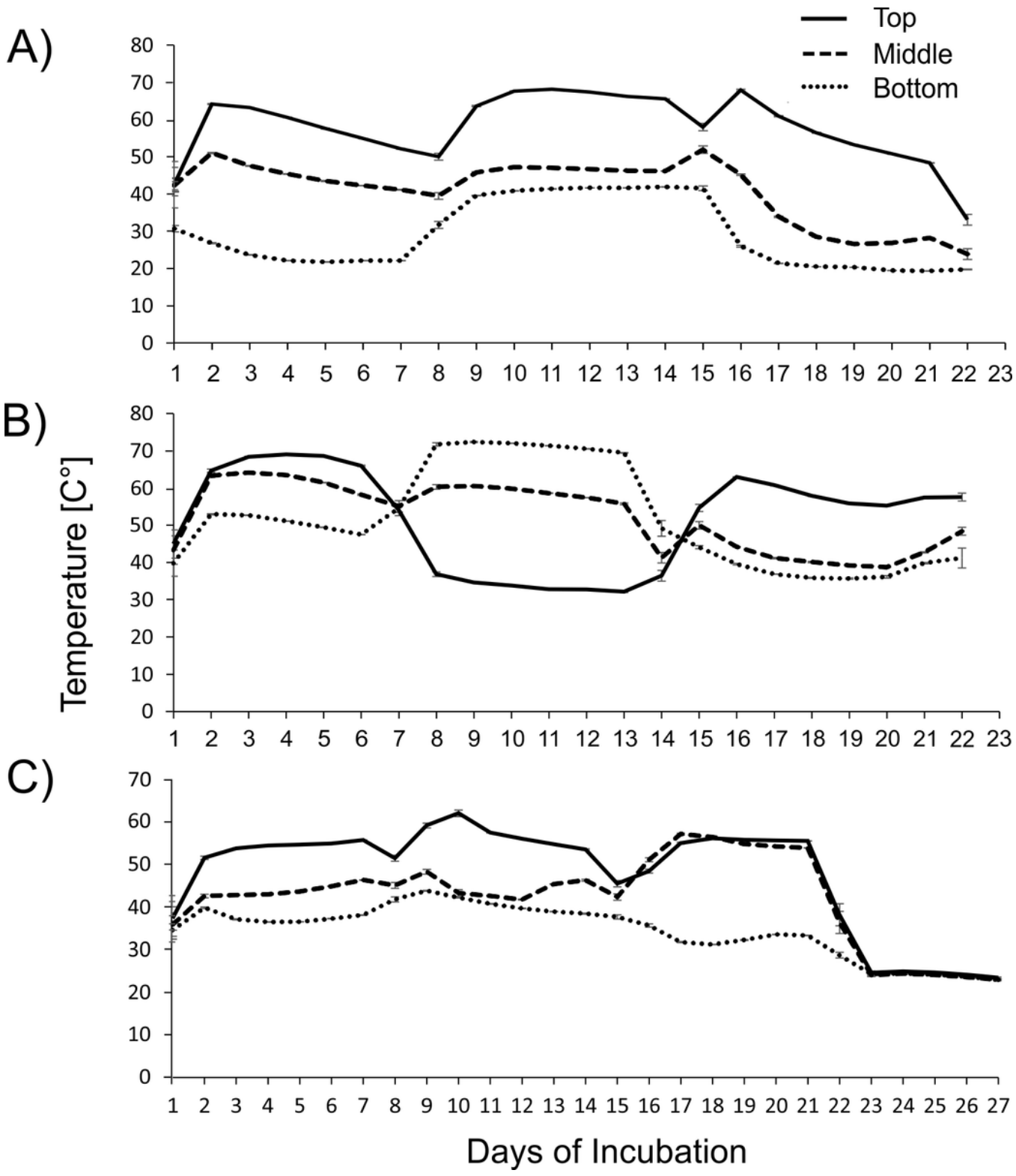

Figure 7

Figure 6: Daily mean temperatures with standard error taken at Top, Middle and Bottom position for three composting runs conducted in (A) autumn 2020, (B) winter 2021, and (C) summer 2021 at the composting plant in Bützberg

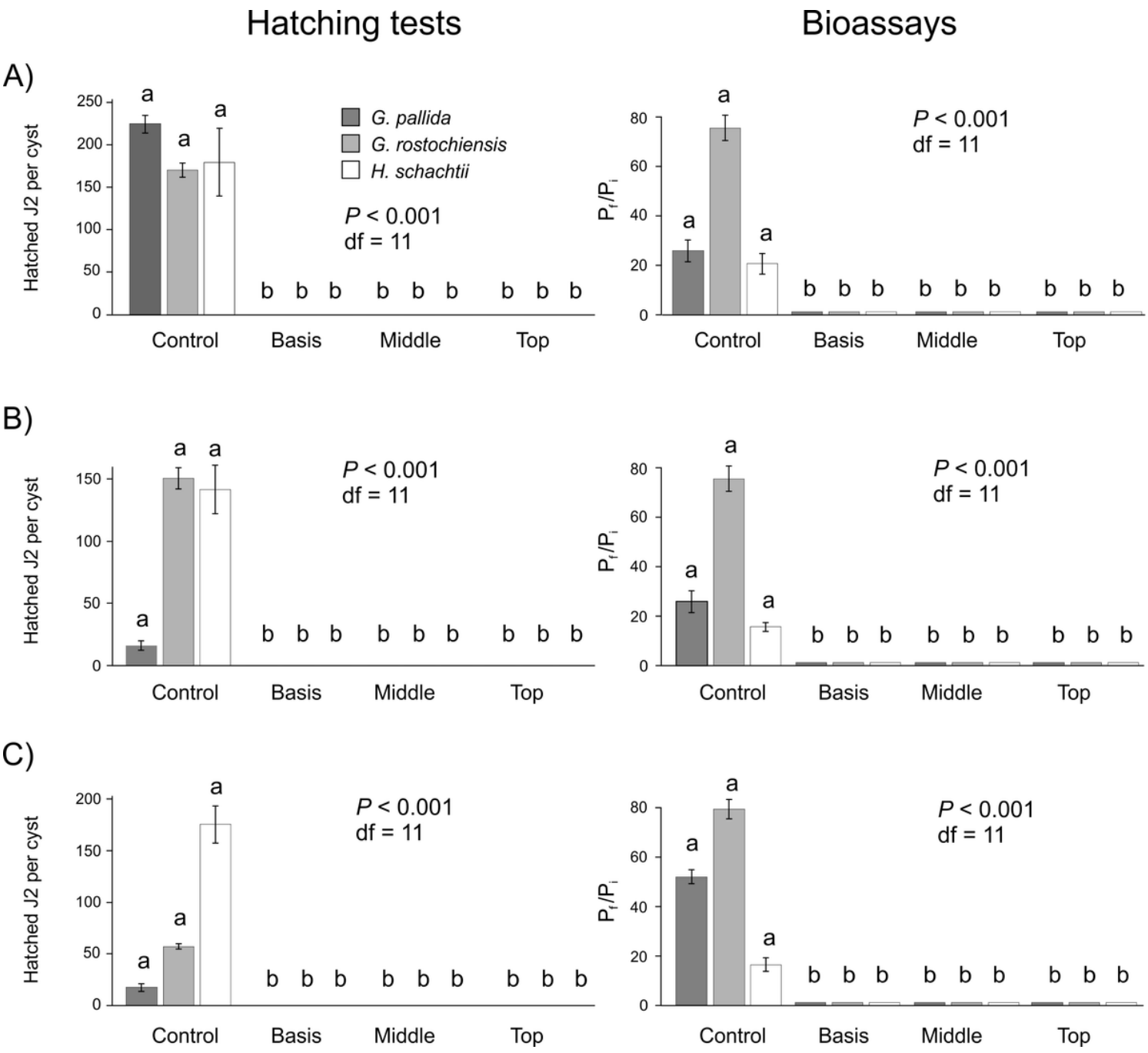

Figure 8

Figure 7: Number of hatched juveniles in the second stage of nematode development shown as the number of hatched juveniles per cyst (hatching tests) and reproduction of adult female nematode cysts (bioassays) conducted on host plants of *Globodera* spp. and *Heterodera schachtii*. Results show composting runs at the different positions of the hybrid-fermenter (n = 4 for each of the positions) under aerobic conditions in A) autumn 2020, B) winter 2021, and C) summer 2021. Standard errors are shown

in whiskers. Statistical differences were calculated using Kruskal-Wallis tests for the hatching tests and bioassays and differences between the sub-groups were calculated by Dunnett's post-hoc test and are presented using different lower case letters above each bar graph

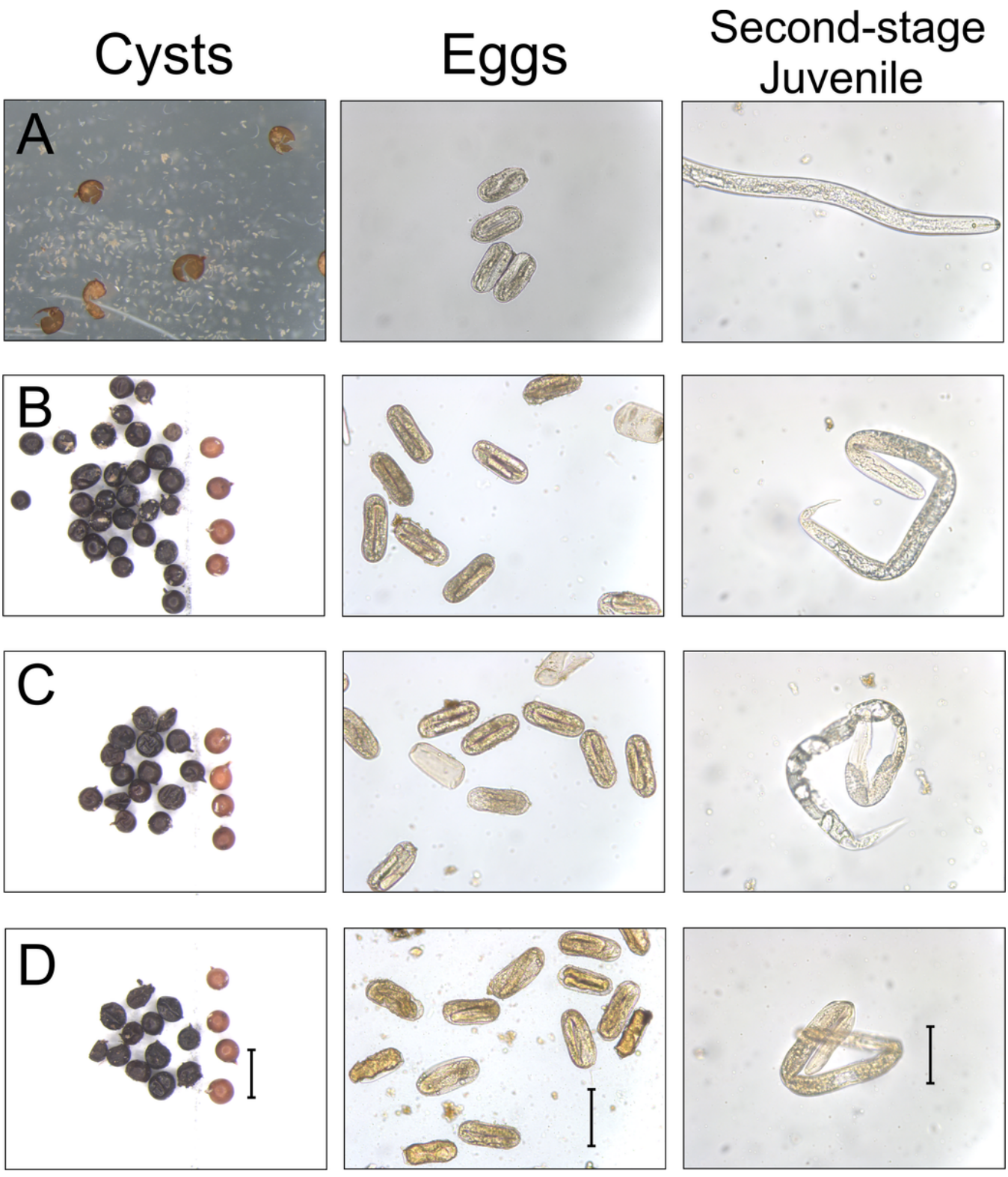

Figure 9

Figure 8: A) Untreated *Globodera pallida* cysts, eggs and juveniles. B-D) Treated *G. pallida* cysts including four reference cysts, distinguishable by their light brown colour, for comparison B) eggs and hatched juveniles treated by anaerobic fermentation. C) *G. pallida*cysts, eggs and juveniles after aerobic composting in the hybrid fermenter D) *G. pallida* cysts, eggs and juveniles after anaerobic fermentation and subsequent composting. Scale bars shown under D) measuring 1 mm (left) under 20x magnification and 100 µm under 400x magnification (middle and right side).